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(54) Title: METHOD OF TREATMENT OF MAMMALS USING ANTIBODIES TO EARLY PREGNANCY FACTOR (EPF)

(57) Abstract

Monoclonal and polyclonal antibodies, and active fragments thereof, to EPF can be used to identify tumour cells which produce EPF to provide an early monitor for cancer in mammals. The tumour cells can be destroyed in the mammal by administration of the antibodies, or fragments thereof, to EPF to the mammal. The antibodies are useful in detecting the presence of EPF in serum during pregnancy and provide a means for detecting and, if required, terminating pregnancy in mammals.

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Title: "METHOD OF TREATMENT OF MAMMALS USING ANTIBODIES TO EARLY PREGNANCY FACTOR (EPF)"

TECHNICAL FIELD:

This invention relates to methods of treatment of mammals, including human beings, with antibodies or active fragments thereof, to early pregnancy factor (EPF). These antibodies are of particular use in the detection and destruction of tumours. They also find use as a means to detect and, if necessary, to terminate pregnancy.

BACKGROUND TO THE INVENTION:

Early pregnancy (EPF) is a protein which has been detected in the serum and urine throughout the first and second trimesters of pregnancy in mammals, the initial and continuing production of EPF being dependent upon the presence of a viable embryo. A method of producing a monoclonal antibody which can be used to detect the presence of EPF, and thus act as a pregnancy diagnostic agent, has been developed by the present inventors and is the subject of Australian Patent Application No. 55897/86.

Although a bioassay for the detection of EPF in cells is also known - the modified rosette inhibition test - it is an indirect assay and is not quantitative in its detection of EPF. Further, the inhibition test is tedious and time-consuming to undertake and is not a suitable assay to be packaged as a routine laboratory test for identification of tumour cells.

As transformed and neoplastic cells in the active growing phase also produce EPF, it would obviously be an advantage if these tumour cells could be easily detected and/or specifically targeted for destruction.

DISCLOSURE OF THE INVENTION

The present inventors have now discovered that

monoclonal and polyclonal antibodies, or active fragments thereof, to EPF can be readily used to identify and, more importantly, destroy tumour cells.

The antibodies and active fragments may be produced by any suitable technique known in the art, and may be produced against EPF derived from any source or by any means, including recombinant DNA technology, as well as against synthetic peptides corresponding with the appropriate part of the amino acid sequence of EPF.

Thus, according to a first aspect of the present invention, there is provided a method of identifying tumour cells, said method comprising:

(1) adding to the serum of a patient, an antibody, or active fragment thereof, to EPF; and

(2) monitoring any reaction due to the presence of EPF.

Both monoclonal and polyclonal antibodies, or active fragments thereof, to EPF can be used, although monoclonal antibodies are preferred.

In a second aspect of the present invention, there is provided a method of destroying a tumour cell in a patient, said method comprising:

administering to said patient an antibody, or active fragment thereof, to EPF in sufficient quantity to destroy said tumour cell.

Experiments were conducted in which mice were injected subcutaneously with tumour cells and, at the same time, with an anti-EPF antibody. In these mice, tumours failed to develop. Tumours developed in the control group of mice which received injections of the tumour cells but no anti-EPF antibody.

Similarly, tumour cells failed to grow in culture in the presence of an anti-EPF antibody.

It will be appreciated that, if the patient is a female of offspring bearing age, it will be necessary

to undertake a further test to ensure that any positive reaction to an EPF antibody is not due to pregnancy.

However, a further advantage of the present invention is that the polyclonal antibodies to EPF, either in isolation or in combination with monoclonal antibodies to EPF, can be used in a pregnancy test for mammals, including humans.

Thus according to a third aspect of the present invention, there is provided a method for pregnancy diagnosis, said method comprising:

(1) mixing a polyclonal antibody, or active fragment thereof, to EPF with serum, blood or urine from the mammal believed to be pregnant; and

(2) monitoring the reaction to determine if EPF is present in the serum, blood or urine.

As urine from a mammal can be collected e.g. from the floor of an enclosure, no human handling of the mammal need be required and this is important when testing endangered species which are adversely sensitive to human contact such as the Chinese Giant Panda.

In a fourth aspect of the present invention there is provided a method of terminating pregnancy in a mammal, said method comprising:

administering an antibody, or active fragment thereof, to EPF to the mammal to remove the EPF from the mammal's serum.

When the EPF level drops, the mammal will abort the foetus, terminating the pregnancy.

As the lack of EPF prevents the development of the fertilized ovum, the EPF antibody could be incorporated into a "morning after" treatment or could be used later in gestation.

In experiments with mice, the females were injected with an EPF antibody in the period 32-56 hours

after confirmed mating (in the early pre-implantation stage). In a significant number of cases, no pregnancies occurred, indicating that the pregnancies had been aborted.

Preferably, in all appropriate embodiments of the present invention, the reaction is monitored by using either a second polyclonal or monoclonal antibody or active fragment thereof.

DETAILED DESCRIPTION OF EMBODIMENTS:

Monoclonal antibodies to EPF were prepared as described in Australian Patent Application No. 55897/86, from mice immunized with EPF, purified from medium conditioned by the choriocarcinoma cell line BeWo (ATCC deposit No. CCL22). Since the planned experiments included neutralisation of mouse EPF in vivo, immunised mice with high titre antibodies to mouse pregnancy EPF were selected. Fused cells were selected by growing in medium containing hypoxantine, aminopterin and thymidine (HAT medium) and spent medium from the growing hybridomas was screened for anti-EPF immunoglobulin (Ig) production. However, as observed with the parent myeloma cells (as with all cancer cells), hybridoma cells in culture produce EPF; thus specific antibody-secreting hybridomas produce both EPF and anti-EPF. Therefore, before testing for anti-EPF antibodies, culture supernatants must be treated to remove EPF, both free and bound to antibody.

Medium (20ul) was acidified to pH 2.5 with 1 ml 0.2 M glycine-HC1, pH 2.0, mixed at room temperature for 0.5 h, then passed through a Waters C_{18} Sep-pak (Waters Ass, Ma, USA). The Sep-pak had previously been activated with 5 ml acetonitrile (Mallinckrodt, Ke, USA), washed with distilled water and equilibrated with glycine-HC1 just before use. The flow-through fraction was collected, pH raised to 8.0 with solid

Tris (Sigma), dialysed against PBS and then tested anti-EPF specificity as follows. A 0.1 ml sample of treated supernatant, 1:1 00 in PBS, was incubated at 37°C for 0.5 h with 0.1 ml purified mouse EPF, 500 pg ml^{-1} in PBS. After incubation, the mixture was tested for EPF activity in the rosette inhibition test.

Ig that was found to be EPF-specific was tested for class and sub-class by a solid phase immunoassay with sheep anti-mouse IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 (Nordic, Tilburg, Netherlands; 1:4 000 in binding buffer) bound to the tubes. Two vigorously growing clones 7/342 and 5/341 were selected and maintained by growing hybridoma cells as ascites in male BALB/c mice; both clones produced IgM. A control preparation of IgM was prepared from a further clone 7/331, producing IgM, but not of anti-EPF specificity. Hybridomas (10^6 cells per mouse) were injected into 12 week old BALB/c mice, primed with pristane (2,6,10, 14-tetramethylpentadecane; Sigma) 7 d before injection of cells. Ascitic fluid was drained, allowed to clot and centrifuged at 3 000g, 4°C for 20 min, after which the fat was removed by aspiration. The pH of the ascitic fluid was adjusted to pH 2.5 with glycine -HC1 H 2.0, then passed through a C_{18}Sep-pak cartridge to bind EPF. IgM in the flow-through fraction was precipitated by dialysis against 5 mM Tris-HC1, pH 7.5, the precipitate washed x 2 in dialysis buffer, then reconstituted in PBS/0.05% sodium azide and stored at 4°C. SDS-PAGE was performed in horizontal 250 x 110 x 0.5 mm gels with an exponential acrylamide gradient from 4 to 22.5%, prepared in 0.375 M Tris-HC1, pH 8.8/0.1% SDS and run in 0.375 m Tris-glycine, pH 8.3/0.1% SDS. Samples were diluted with an equal volume of

0.075 M Tris-HCl. pH 8.8/2% SDS/0.1 M DTT, then boiled for 3 min and cooled to room temperature before application of 2 ul to the gel. Electrophoresis was performed for 1.75 h at 600 V, then the gel fixed for 30 min in 20% trichloroacetic acid (TCA), stained overnight in 15% TCA containing 0.05% Coomassie Brilliant Blue R250 (LKB, Bromma, Sweden) and destained in 12.5% TCA. Results confirmed that monoclonal antibodies 7/342, 5/341 and 7/331 are IgM, with heavy and light chains demonstrated at approximate M_r 75 000 and 25 000 respectively, and showed that the preparations were virtually free from contaminants.

Polyclonal antibodies were prepared by immunizing host animals (e.g. rabbits, donkeys or other suitable hosts) with EPF purified from a variety of sources [e.g. medium conditioned by the choriocarcinoma cell line, BeWo (ATCC Deposit No. CCL98), Madin Darby bovine kidney cell line MDBK (ATCC Deposit No. CCL22)]. Purified EPF (5 ug - 30 ug) was emulsified in Freund's complete or incomplete adjuvant and injected into animals at monthly intervals. Starting after the fourth injection, serum from the immunized animals was tested for antibodies capable of binding to EPF in a solid phase immunoassay. Suitable antibodies were purified from serum by sodium sulphate precipitation followed by chromatography on DEAE Affi-Gel Blue (Bio-Rad Richmond, California, U.S.A.). Preparations from rabbits ##7 and ##8 were used in the experiments. SDS-PAGE was performed on these preparations as previously described and the results confirmed that the antibodies were IgG, with heavy and light chains demonstrated at approximately M_r 55,000 and 25,000 respectively and virtually free from contaminants. Both these preparations, as well as the monoclonal preparations 7/342 and 5/341, neutralized the

activity of purified EPF, e.g. 500 pg (0.04 pmol) EPF was neutralized by 5 ng poluclonal (0.03 pmol) and 50 ng monoclonal antibodies (0.05 pmol) but not by 1 ng (0.006 pmol polyclonal nor 5 ng (0.005 pmol) monoclonal. The monoclonal antibodies, 7/342 and 5/341 have been shown to bind to different epitopes of the EPF molecule. A control monoclonal antibody 7/331, not of anti EPF specificity, was prepared for use in the experiments described below. It did not neutralize EPF at any concentration.

Experiments have shown that transformed and neoplastic cells in the active growing phase produce EPF, with production ceasing after growth arrest or cell differentiation. Experiments have shown that monoclonal anti-EPF antibodies can adversely affect the growth of these tumour cells both in culture and after transplantation into mice as solid tumours. A mouse methylcholanthrene - induced fibrosarcoma cell line (MCA2) has been used in both in vivo and in vitro experiments and a mouse melanoma cell line B16 in an in vitro study.

The results of these experiments are graphically illustrated in Figures1 and 2.

Two monoclonal anti-EPF antibodies, 7/342 and 5/341, have been used in the in vitro experiments.

Tumour cells (10^4) in 2 mls medium (Dulbeccos modification of Eagle's medium) with foetal calf serum were seeded into each petri dish and varying doses of 7/342 antibody added to give final concentrations ranging from 1 uM to 0.05 uM (6 different concentrations tested in triplicate). Cells were microscopically examined 48 hours after the addition of antibody and no difference was observed between cells incubated with anti-EPF 7/342, control antibody 7/331 or without antibody. However, after 96 hours there was a marked

difference in the appearance of cells incubated with 7/342; in the higher antibody concentrations the cells were detached and clumping. The viability of the cells was tested, 24 hours after the addition of antibody, by ^{3}H-thymidine incorporation and trypan blue exclusion. The effect of the antibody on the viability of cells was dose dependent, with 50% non-viable cells at 0.16 uM concentration of antibody, see Figures. There was no difference in ^{3}H-thymidine uptake of cells incubated with 7/331 control antibody in concentrations the same as those used for 7/342. The viability of fresh spleen cells, incubated in parallel, was not affected by either antibody. Similar results were obtained with anti-EPF monoclonal antibody 5/341, known to bind to a different epitope of the EPF molecule from that binding 7/342.

In vivo studies have also been carried out. Preliminary tests established that following subcutaneous injection of 10^{6} MCA-2 cells into CBA mice, tumours were palpable within seven days (3.0 mm SD 0.8, n=4). By 10 days, the tumours increased to 5.7 mm (S.D. $\pm$ 1.2) and by 13 days to 13.0 mm (S.D. $\pm$ 2.8). In the experiments with anti-EPF 7/342, 10^{6} MCA-2 cells were injected subcutaneously and various doses of antibody administered at different times; 1 mg 7/342 injected at the time of inoculation with MCA-2 cells did not affect the growth of the tumours; however, mice receiving 500 ug daily for 4 days did not develop tumours by day 13. These results indicated that the growth of tumour cells can be significantly retarded by anti-EPF antibodies.

Outbred female Quackenbush mice were caged overnight with males. Pregnancy was dated from the morning of detection of a vaginal plug (day 1) and time of mating assumed to be 2.00 a.m. on this day. Serum for detection of EPF by the rosette inhibition test

(the bioassay for EPF) was taken from the mated females, before administration of the first dose of antibody. To confirm that a positive assay for EPF in serum at 24 hours was evidence of pregnancy, a group of 23 mated mice was tested for EPF at 24 hours and examined for embryos 10 days later. Of the 15 mice in this group that were pregnant at 10 days, 14 gave a positive assay for EPF at 24 hours, and one negative. The 8 mice that were not pregnant at 10 days all gave a negative assay at 24 hours. Thus only mice positive for EPF in the bioassay were included in the study.

In the first experiment, mice received 2 injections i.p. of a monoclonal anti-EPF antibody 7/342 at 32 and 56 h post-coitus, in doses ranging from 64 ug to 500 ug per injection. Control groups received 1) mouse monoclonal antibody of specificity other than anti-EPF and 2) 0.9% NaCl, following the same protocol (Table 1).

In a second experiment mice received four injections of polyclonal anti-EPF antibodies ~~#7~~ and ~~#8~~ (500 ug per injection) at 8, 16, 32 and 40 hours p.c. Control groups received 1) normal rabbit IgG following a similar protocol or 2) 0.9% NaCl. Mice were killed 7 d post-coitus and the number of embryos counted (Table 2).

TABLE 1

The effect on foetal viability of passive immunisation of confirmed-mated mice with anti-EPF monoclonal antibodies (Mab)

Injection	Group	Route of Injection	Dose Mab (ug) x 2	No.pregnant at 10 d/ No. treated	No. of embryos/ mouse
7/342 (anti-EPF)	1.	i.p.	400	1/3^{+}	2
	2.	i.p.	250	1/5*	13
	3.	i.p.	125	2/5^{+}	7, 13
	4.	i.p.	64	1/3^{+}	12
7/331	5.	i.p.	250	5/5^{o}	13.2 ±1.6(5)
(Control)	6.	i.p.	125	4/4^{o}	12.3 ±0.8(4)
0.9% NaCl	7.	i.p.	---	17/18	14.5 ±0.4(17)

In the test group, the number of embryos/mouse are listed. In the control groups, the results are given as mean ±s.e.m. (numbers of animals are shown in parentheses). The number of mice pregnant at 10 d, in each group of 1 - 6 receiving Mab, was compared with the number in group 7 receiving 0.9% NaCl (X^2 test);
* $p < 0.001$, $^{+}p < 0.01$, ^{o}p=NS

TABLE 2

The effect on foetal viability of passive immunization of confirmed-mated mice with anti-EPF polyclonal antibodies #7 and #8.

Antibody Injection Commencing 8 h p.c	Dose Per Inject.	No of Injects.	Mice Pregnant at 7 d/ Mice Treated	No. of Embryos
Polyclonal Rabbit Anti-EPF IgG				
1. #7	500µg	4	2/6+	11, 15
2. #8	500µg	4	1/6*	8
Control				
3. Rabbit IgG	500µg	4	4/4	15, 12 16, 17
4. Saline	-	4	9/9	14.5 +0.31 (s.e.m.)

+1. v 4 $p < .01$ (χ^2 test) * 2. v 4 $p < .001$ (χ^2 test)

Various changes and modifications may be made to the embodiments described without departing from the present invention as defined in the following claims.

CLAIMS:

1. A method of identifying tumour cells, said method comprising:

(1) adding to the serum of a patient, an antibody or active fragment thereof, to EPF; and

(2) monitoring any reaction due to the presence of EPF.

2. A method as defined in Claim 1, wherein said reaction is monitored by using either a second polyclonal or monoclonal antibody or active fragment thereof.

3. A method as defined in Claim 2, wherein said antibody is a polyclonal antibody and said reaction is monitored by using a monoclonal antibody.

4. A method as defined in any one of Claims 1 to 3, wherein the antibody or active fragment thereof is produced against EPF derived by recombinant technology or is produced against synthetic peptides corresponding with the appropriate part of the amino acid sequence of EPF.

5. A method as defined in any one of Claims 1 to 4, wherein the antibody is the antibody 7/342 as herein described.

6. A method as defined in any one of Claims 1 to 4, wherein the antibody is selected from the polyclonal antibody ##7 or polyclonal antibody ##8 as herein described.

7. A method of destroying a tumour cell in a patient, said method comprising:

administering to said patient an antibody, or active fragment thereof, to EPF in sufficient quantity to destroy said tumour cell.

8. A method of pregnancy diagnosis, said method comprising:

(1) mixing a polyclonal antibody, or active fragment thereof, to EPF with serum, blood or urine from the mammal believed to be pregnant; and

(2) monitoring the reaction to determine if EPF is

present in the serum, blood or urine.

9. A method as defined in Claim 8, wherein said reaction is monitored by using either a second polyclonal or monoclonal antibody or active fragment thereof.

10. A method of terminating pregnancy in a mammal, said method comprising:

administering an antibody, or active fragment thereof, to EPF to the mammal to remove EPF from the mammal's serum.

Figure 1

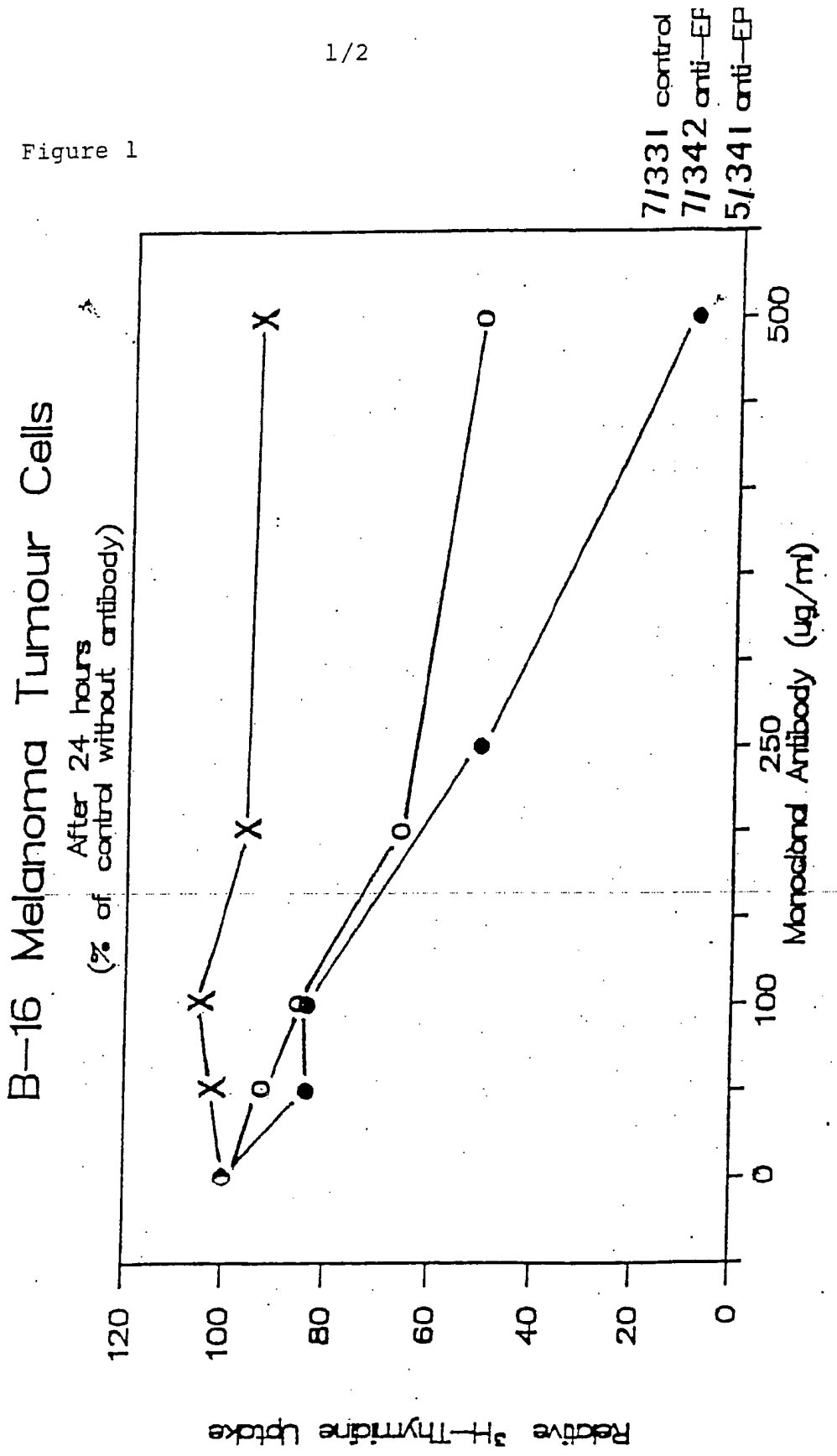
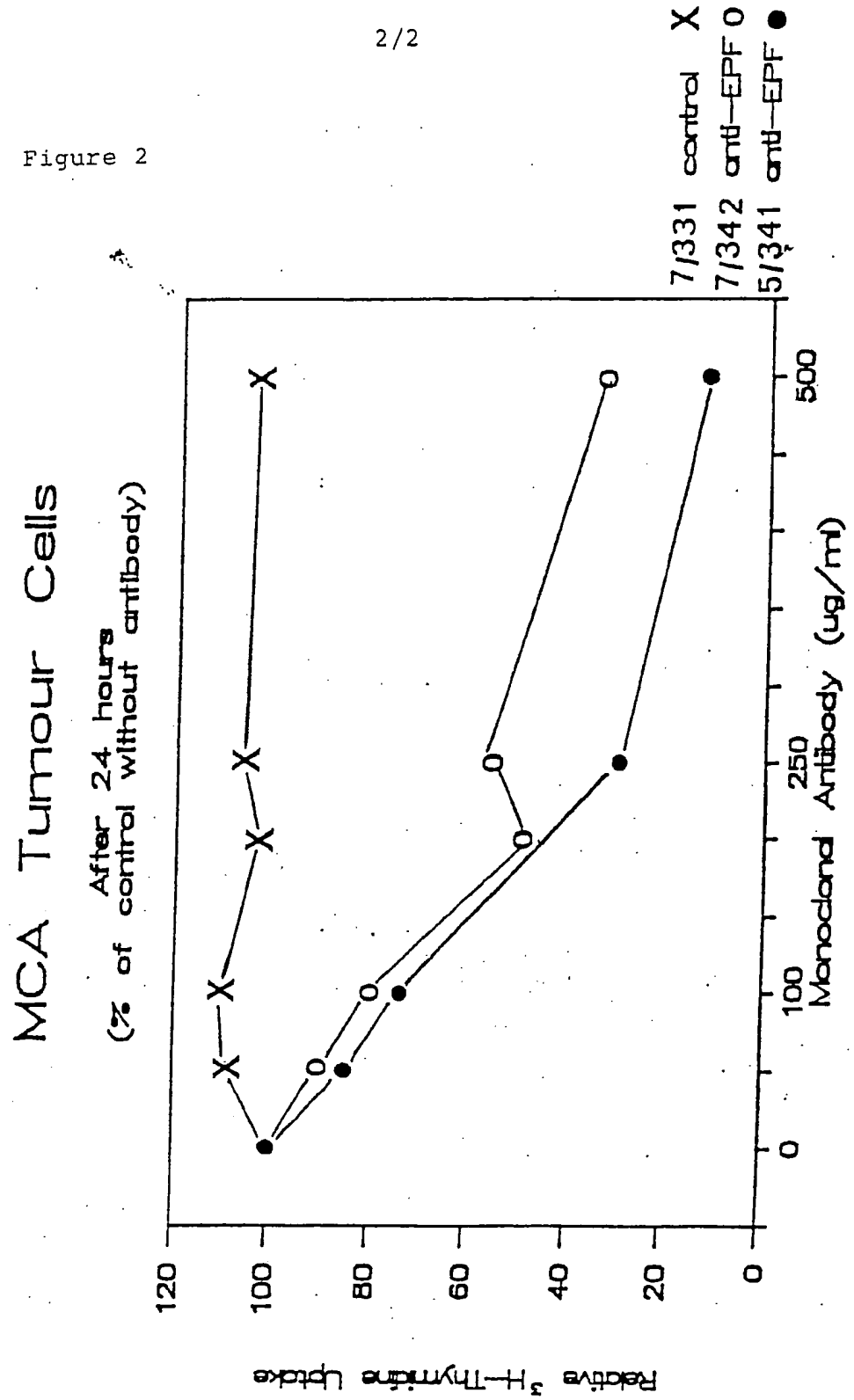


Figure 2


MCA Tumour Cells
After 24 hours
(% of control without antibody)
7/331 control X
7/342 anti-EPF O
5/341 anti-EPF ●
Relative ³H-Thymidine Uptake
Monoclonal Antibody (ug/ml)
0
20
40
60
80
100
120
0
100
250
500

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00432

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] G01N 33/574, 33/577, 33/74, A61K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched †

Classification System	Classification Symbols
IPC	KEYWORD SEARCH 'EARLY PREGNANCY FACTOR'

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ‡

AU : IPC G01N 33/74, 33/574, 33/54

III. DOCUMENTS CONSIDERED TO BE RELEVANT §

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ††	Relevant to Claim No. ††
X,Y	WO,A, 86/05498 (UNIVERSITY OF QUEENSLAND) 25 September 1986 (25.09.86)	(1-10)
Y,P	American Journal of Reproductive Immunology and Microbiology, Volume 14 No.3, issued 1987, March (New York) A.R. Mehta et al, "Detection of Early Pregnancy Factor-like Activity in Women with Gestational Trophoblastic Tumours", see pages 67-69	(1-7)
Y,P	Journal of Reproductive Immunology, Volume 10 No.2, issued 1987, April (Amsterdam) F.M. Clarke et al, "Early Pregnancy Factor: large scale isolation of rosette inhibition test-active polypeptides from ovine placental extracts", see pages 133-156	(8-10)
Y	Journal of Reproductive Fertility, Volume 71 No.2, issued 1984 (Great Britain) A.C. Cavanagh, "Production in vitro of mouse early pregnancy factor and purification to homogeneity", see pages 581-592	(8-10)

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31 March 1988 (31.03.88)	06 APRIL 1988 (06.04.88)
International Searching Authority: Australian Patent Office	Signature of Authorized Officer: Kim Sach

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00432

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members	
WO 8605498	AU 55897/86	GB 2192634

END OF ANNEX